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Sponges of the Danube area

Contribution to the knowledge on the distribution of freshwater sponges – the Danube and Sava rivers case study

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ABSTRACT

Sponges in the large rivers within the Danube River Basin (DRB) have not been adequately studied. Hence, the aim of this work was to undertake an investigation on the distribution of sponge species in the Danube and Sava rivers. Out of 88 localities covered by the study, sponges were found at 25 sites only (46 samples in total). By using morphological (light and scanning electron microscopy) and genetic (28S rDNA sequencing) analysis, four species were

determined: *Ephydatia fluviatilis* (Linnaeus, 1759), *Spongilla lacustris* (Linnaeus, 1759), *Eunapius fragilis* (Leidy, 1851), *Trochospongilla horrida* Weltner, 1893. In the Danube, the predominant species was found to be *E. fluviatilis* making approximately 80% of collected samples, while in the Sava River *S. lacustris* dominated, representing 46% of the river sponges. Our work represents one of the few studies on freshwater sponges within the DRB from long stretches of the large lowland rivers (more than 2500 km of the Danube River and about 900 km of the Sava River). Moreover, molecular analysis for the identification of freshwater sponges was applied on the material collected from a wide area, thus contributing to the systematic studies on the distribution and abundance of the European freshwater invertebrate fauna in general.

INTRODUCTION

Sponges (Porifera) constitute one of the most diverse metazoan phyla, with more than 8500 known species (Van Soest *et al.*, 2012) and about 17,000 to 29,000 yet to be described (Itskovich *et al.*, 2013; Morrow *et al.*, 2013). Though the great majority of sponge species inhabit marine ecosystems, 237 species from 47 genera grouped in 7 families of the order Spongillida (Manconi and Pronzato, 2002) can be found in freshwater environments, the Neotropical region showing the highest richness in species, followed by Palearctic (Manconi and Pronzato, 2002, 2016; Manconi *et al.*, 2013). Sponges are sessile organisms that represent an important component of aquatic ecosystems, with a significant filtering potential important for natural processes of water purification. They filter particles of a smaller size than other benthic invertebrates (Frost, 1978; Francis and Poirrier, 1986), ranging from zoo-phyto-pico plankton to bacteria (Manconi and Pronzato, 2015). Since they populate a diverse array of habitats and thrive in waters with different levels of pollution they may be used as water quality bioindicators (Rao *et al.*, 2009). Some papers suggest their applicability in the measurement of levels of heavy metals in the water (Richelle, 1995). Their fossil spicules found in the sediment are a useful indicator of paleoenvironmental changes (Manconi and Pronzato, 2015). They have also proved to be valuable objects for biodiversity monitoring as suggested for freshwater environments of South America (Volkmer-Ribeiro and Machado, 2007) and Africa (Manconi and Pronzato, 2007). They are also of great interest for the pharmaceutical industry as they contain various bioactive compounds with anti-tumor, anti-infective and anti-inflammatory properties (Van Soest *et al.*,

2012). The biochemical pathways of synthesis of these compounds are often unknown (Roovere *et al.,* 2006) and since sponges can be grown and develop *in vitro* from their resting bodies (gemmules), they are a good experimental model for studying these biochemical pathways as well (Lopp *et al.,* 2007).

Studies on freshwater sponges in Europe have been marked by periods of essential discoveries with intermissions of lesser interest. Among the first studies on European Porifera were those by Annandale conducted in Scotland at the beginning of the 20th century. The knowledge on sponges in Europe has been later considerably broadened by contributions of Arndt (1926, 1931, 1932a, 1932b). In more recent decades important papers have been published covering a number of European countries and their freshwater systems: Denmark and Iceland (Tendal, 1967a, 1967b, 1976), Romania (Rudescu, 1975), Belgium (Richelle-Maurer *et al.*, 1994), Switzerland (Manconi and Desqueiroux-Faundez, 1999), Spain (Traveset, 1990), Italy (Manconi and Pronzato, 1994), Norway (Økland and Økland, 1996), Germany (Gugel, 2001), Estonia (Roovere *et al*., 2006), Austria (Dröscher and Waringer, 2007), etc. More specifically, when considering inland waters of Central Europe, six sponge species have been recorded: *Ephydatia fluviatilis* (Linnaeus, 1759), *Spongilla lacustris* (Linnaeus, 1759), *Eunapius fragilis* (Leidy, 1851), *Trochospongilla horrida* Weltner, 1893, *Ephydatia muelleri* (Lieberkühn, 1856) and *Heteromeyenia stepanowii* (Dybowski, 1884) (Gugel, 2001; Dröscher and Waringer, 2007).Interestingly, the Balkan Peninsula, drained by many rivers and generally characterized by great diversity and complexity of aquatic fauna (Bănărescu, 2004), has not been, with few exceptions, systematically screened for the presence of freshwater sponges. Besides the capital work of Rudesku (1975) on Porifera Potamospongiae in Romania, few studies have been published from this area. For instance, a study on sponges from large Macedonian lakes Prespa, Dojran and Ohrid, has reported on *Eunapius carteri* (Bowerbank, 1863), *S. lacustris*, *E. fluviatilis*, *E. fragilis*, *Spongilla prespensis* Hadzische, 1953, *Spongilla stankovici* Arndt, 1938, *Ochridaspongia rotunda* Arndt, 1937 (Hadzisce, 1953), or on a troglobiotic freshwater sponge found in the karst of the Dinarid region of Croatia *Eunapius subterraneus* Sket & Velikonja, 1984 (Bănărescu, 2004; Bilandžija *et al.*, 2007).

Regarding Sava and Danube, which belong to both Central Europe and the Balkans, data are again very limited. Several older (Matoničkin *et al.*, 1975; Mihaljević *et al.*, 1998) and more recent (Graf 2015, Lucić 2015) comprehensive and large-scale surveys of Sava and Danube

rivers have been conducted, dealing with various aspects of their ecology. These studies included an in-depth analysis of macroinvertebrates pointing again to the taxa richness, but with extremely scarce data, if any, on freshwater sponges. Just recently Anđus *et al.* (2016) gave preliminary findings on *S. lacustris* and *E. fluviatilis* in the Serbian stretch of the Danube. Also, in the Danube basin management plan of Croatia, several sponge species have been listed, without further analysis (*E. fragilis*, *E. carteri*, *E. fluviatilis, E. Muelleri, S. lacustris, E. subterranea* (Croatian Water Management Plan, 2013).

This prompted us to conduct the present study with the aim to contribute to the knowledge on Porifera distribution along considerable stretches of the Danube (2580 km) and Sava (900 km) rivers, with emphasis on less explored areas. For higher accuracy of species identification, in addition to the classic approach that relies on morphological characters, genetic analysis was also used.

METHODS

Basic physical and chemical properties such as pH, temperature, dissolved oxygen and conductivity were determined on the spot, using HANNA HI 9126 pH, HI 9146, and HI98130 instruments, and TFA EN 13485 Digital thermometer. The concentration of nitrates, phosphates and ammonium salts were measured from water samples brought to the laboratory, using WTW chemical kits as recommended by manufacturer, and WTW Photo Lab Spectral spectrophotometer.

Sponge samples were collected during chemical and biological monitoring of the Sava and Danube rivers in the framework of GLOBAQUA project (Navarro-Ortega *et al.*, 2015), and Joint Danube Survey 3 investigation (Graf, 2015) respectively in the period 2013-2015. A total of 68 locations were explored on the Danube River, and sponges were found at 17 of them. The Sava River was studied at 20 locations, from Slovenia to Serbia, and samples were found and collected at 7 sites. Potential finding sites, characterized by reduced flow, rocky bed and wood debris, referred to as "characteristic habitats", were visually inspected either from a boat, by wader walking or free diving. Samples were collected at depths between 0-5 m on river stretches of about 100 by 1 m along the river side. About 50 rocks, branches or other submerged objects were inspected per locality. Fragments from each sample were taken for spicule preparation and genetic analysis. An initial assessment of sample preservation quality in different media was

done (4 % formaldehyde, 70 % ethanol, and dried). The type of preservation had no impact on spicule preparation, while the quality and yield of DNA was greater in samples stored in 70% ethanol, which led to later use of ethanol only.

For the assessment of relative abundance (substrate coverage) of sponges, an adaptation of the approach suggested by Dorschner *et al.*, (1993) was implemented. In brief, the following criteria were applied:

"small single colonies" (level 1): only one or few specimens found within transecton site;
"several colonies" (level 2): ≤10 specimens per transect;
"numerous colonies" (level 3): more than 10 specimens per transect. No greater coverage was registered.

The nitric acid technique as described by Manconi and Pronzato (2015), was used to dissolve sponge tissue and prepare spicules for light microscope analysis. Briefly, 2-5 mm sponge fragments were washed with ethanol, dried and fed into labeled glass tubes. They were then carefully topped with 2-5 mL of concentrated nitric acid (HNO_3) and left to decompose for 24 h. The acid was then removed by pipette and the spicule residues were washed repeatedly with distilled water. Finally, the spicules were rinsed with and resuspended in 96 % ethanol. A drop of suspension was then placed on a cover slip. When the alcohol dried the cover slip was placed over the microscope slides with a drop of Canada balsam and heated to complete the preparation. Drops of spicule suspension in ethanol were placed on specimen holders and coated with gold in a gold sputter at 18 mA for 1 min. The specimens were analyzed and photographed in a VEGA TS 5133MM Scanning Electron Microscope (SEM), high vacuum mode using the SE detector with accelerating voltage.

Specimens for the extraction of genomic DNA were air-dried at 56° C for 1 h, and homogenized in 1:5 (weight:volume) lysis solution containing: 4 M guanidine hidrochloride, 50 mM Tris-HCl pH 8.0, 0.05 M EDTA, 20 µg μL^{-1} of proteinase K and 1 % ß-mercaptoethanol. The suspension was incubated at 50° C for 1 h. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and nucleic acids were precipitated from the upper aqueous phase with 2 volumes of ethanol and 1 10^{-1} volume of sodium acetate. The pellet was washed in 70% ethanol, air-dried and dissolved in nuclease-free water.

A fragment of approximately 340 bp (base pairs) corresponding to the D3 domain of sponge 28S rDNA together with the highly conserved region of approximately 150 bp was amplified using

the following pair of primers (forward 5'-GAC CCG TCT TGA AAC ACG GA-3' and reverse 5'-TCG GAG GGA ACC AGC TAC TA-3') as previously described (Roovere *et al.*, 2006; Lopp *et al.*, 2007). The PCR amplifications were performed in 25 µL reaction volumes containing about 100 ng of sponge DNA, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.5 µM of each primer and one unit of Taq polymerase. The DNA was denatured at 95°C for 1 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C, for 1 min, with a final extension at 72°C for 5 min. The presence of PCR products was confirmed by electrophoresis in 8 % polyacrylamide gel.

The amplification products were directly sequenced in forward and reverse directions using the ABI Big Dye Terminator chemistry and an ABI 3500 instrument (Applied Biosystems, Foster City, CA). For the detection of defects and polymorphic sites on the ends of the sequences we used Sequencher 5.4.6. software (Trial free version). Comparison of the obtained sequences with sequences in the GenBank database was performed using the Basic Local Alignment Tool (BLAST), available at http://www.ncbi.nlm.nih.gov. Sequences were aligned using the program Clustal W with the parameters provided in the software package MEGA (Kumar *et al.*, 2016).

The Neighbor-Joining (NJ) tree was obtained using MEGA 7 software (Saitou and Nei, 1987). The percentage of replicate trees in which the associated specimens clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The analysis involved 25 nucleotide sequences with total of 258 bp in the final dataset. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site.

RESULTS

Basic physical and chemical characteristics (such as water temperature, pH, conductivity and dissolved oxygen) of the Danube and Sava Rivers at the explored sites are given in Tab. 1. Biological monitoring of Sava and Danube rivers revealed scarce and uneven distributionof freshwater sponges. Out of 88 inspected localities in total at the two rivers, sponges, all from the family Spongillidae, order Spongillida, class Demospongiae, were found only at 25 (Fig. 1). Localities of abundance level 1 dominated in both rivers. Only one locality on the Danube (Ram, Serbia) and one on the Sava (Županja, Croatia) had abundance level 3. At the inspected 25 sites,

46 samples were collected. Irrespective of the species, sponges populated waters that were warmer than the average onsite temperature, rich in O_2 and higher concentrations of NO_3 (Tab. 1). Artificial banks with large stones proved to be the most favorable substrate for sponge growth as specimens were found mostly in such environments.

Light microscope analysis was used for rapid sponge determination. Three spicule categories were observed: megascleres, the main spicules of the sponge skeletal structure, smaller surrounding microscleres and gemmuloscleres, that ensheath gemmules and represent the most valuable identification element (Penney and Racek, 1968; Manconi and Pronzato 2002). Skeletal structure analysis showed that the most prevalent sponges were *E. fluviatilis* (Linnaeus 1759), *S. lacustris* (Linnaeus 1759) and *Eunapius fragilis* (Leidy 1851). Characteristic skeletal elements are given in Fig. 2 a,b. Aberrant spicules were relatively common. No deviation in the number of anomalies could be registered in sponges collected at different sites. In all analyzed specimens the number of spicule anomalies (Fig. 2 c,d) varied between 5 and 15 among roughly 1000 spicules per slide. The number of spicule anomalies probably reflects the levels of heavy metals, which varied over a wide range in the two rivers (for instance in the Sava: Cd 0.003-0.020; Pb 0.003-0.234; Cr 0.068-0.426; Cu 0.055–0.881; Ni 0.307-1.07; Zn 0.089-8.74; in the Danube: Cd <0.01-0.145; Pb 0.20-8.08; Cr 0.29-6.73; Cu 1.06-9.93; Ni 0.78-24.63; Zn 1.13-12.95; values are given in µg L^{-1}) (Krämer and Gawlik, 2015; Dragun *et al.*, 2015).

For several sponges light microscope analysis of their skeletal elements was complemented with SEM (Fig. 3), which allowed for substructures of gemmuloscleres to be more accurately analyzed (Manconi and Pronzato, 2002, 2015; Anđus *et al.*, 2016). *E. fluviatilis* and *T. horrida* have gemmuloscleres in the shape of "birotules", two circular elements connected at their centers by a shaft. The indented rotules and shaft visibly longer than the width of rotules is typical of *E. fluviatilis* (Fig. 3a). Birotules of *T. horrida* with smooth rotule edges and short shafts can be seen in Fig. 3b. Gemmuloscleres of *E. fragilis,* ranging in size from 95-120 x 9-12 µm are rod-shaped with rounded or pointed tips. They are covered with spines which are often more concentrated distally. Gemmuloscleres of *S. lacustris,* ranging from 50-120 x 5-7 µm are rod-shaped with rounded tips, from almost straight to strongly bent, covered with spines concentrated at the tips (Schletterer and Eggers, 2006). Taxonomy based on morphology was also confirmed by DNA sequencing. Out of 46 samples that were analyzed, 27 gave good quality sequences. Our DNA sequences from specimens of *E. fluviatilis* were 99% identical with those from GenBank (*E.*

fluviatilis DQ454152, Estonia; EF591285, Italy and JN116226, Israel). DNA sequences of species *E. fragilis* matched with GenBank *E. fragilis* (DQ454155, Estonia) and sequences of species *S. lacustris* were 99% identical with sequence of *S. lacustris* (DQ454154, Estonia) (Tab. 2).

The Neighbor-Joining tree inferred with the alignment of the matrix, including 25 nucleotide sequences of the D3 domain (258 bp) of 28S rDNA, revealed three clades within freshwater sponges of above mentioned species, whereas for the out-group the sequence of marine sponge, *Scopalina ruetzleri* (AY561872) from GenBank was used (Fig. 4). While the tree does not carry enough data to show intraspecific relations it is illustrating the genetic differentiation of 3 prevailing species we encountered.

Within the first clade there are two separations. One contains 14 sequences of the species *E. fluviatilis* (eight from Serbia, three from Croatia and a single specimen from Estonia, Italy and Israel). The other separation contains three specimens of species *E. fragilis*, one from Estonia and two from Croatia and they are presented as three different haplotypes. The second clade contains seven specimens of the species *S. lacustris* (four from Croatia, two from Serbia and one from Estonia). The two approaches (microscopy and molecular analysis) used in sponge identification coincided in all cases. The distribution of species was as follows: in the Danube River *E. fluviatilis* was the predominant species making approximately 80% of collected samples, while *S. lacustris* and *E. fragilis* represented approximately 10% of samples each. In the Serbian section of the Danube, on the 5 localities harboring sponges (Ram, Kladovo, Veliko Gradište, Gornji Milanovac, Vinča), only *E. fluviatilis* was found (12 samples collected and analyzed in total). On the Sava River localities, the same 3 species were found, but with a different distribution: *S. lacustris* (46 %) followed by *E. fluviatilis* (37 %) and *E. fragilis* (17 %). Only *S. lacustris* and *E. fluviatilis* have been found in Serbia (Beograd, Novi Beograd, Ostružnica). Županja, Croatia, was the locality with the highest diversity and abundance of sponges. Interestingly, a widely distributed but rare freshwater sponge *Trochospongilla horrida*, was found on one locality only on the Danube River (Hirsova, Romania).

DISCUSSION

Sponges are becoming increasingly popular as biological indicators of water quality but have mostly been used in marine environments (Rao *et al.*, 2009; Anakina, 2010; Batista *et al.*, 2013). Consequently, it would be beneficial for countries/regions interested in their use for bio-

monitoring purposes to have an overview of their distribution and diversity. This study represents a starting point for future large-scale investigations.

Sampling of sponges along the Danube (from Germany to Romania) and the Sava (from Slovenia to Serbia) revealed a relative paucity of species, as well as low abundance in the majority of examined sites. As far as the Danube is concerned, out of six countries encompassed by the present sampling, sponges were found only in three (Germany, Serbia and Romania). As far as the Sava is concerned, sponges were found in two out of three countries (Croatia and Serbia). This might be due to the fact that not all selected and inspected localities were favorable for sponge development. Namely, absence of adequate growth substrate, faster flow, suboptimal physical and chemical parameters, *etc*. (Elexová and Némethová, 2003) on a number of river stretches could have had impact on the findings. Four species (*E. fluviatilis, S. lacustris*, *E. fragilis* and *T. horrida)*, out of the 6 species recorded in Central Europe were found. Literature data suggests that species belonging to *Ephydatia, Spongilla*, and *Eunapius* are widely distributed (Manconi and Pronzato, 2002, 2008; Manconi *et al.*, 2013), and our findings support this view. In the present study, the same species were found both in the Danube and the Sava Rivers, with the exception of *T. horrida* collected at one site only in the Romanian stretch of the Danube. The most prevalent sponge in this river was *E. fluviatilis*, while in the Sava it was *S. lacustris*. *E. fragilis* was rare in both rivers. Remarkably, at the inspected localities in Austria, no sponges were found. This is in contrast with the results of Dröscher and Waringer who found 5 species in the Danubian floodplain waters near Vienna (*E. fluviatilis*, *S. lacustris*, *E. muelleri*, *E. fragilis* and *T. horrida*). It must be emphasized however, that our sampling did not include the area around Vienna, which may explain data discrepancy. These authors also noted that *E. fluviatilis* favored water temperatures over 21°C, which is in agreement with our findings regarding temperatures characterizing sponge habitats. From its source in the Slovenian mountains to its mouth into the Danube in Serbia, with its total length of 944 km and total catchment area of 97,713 km^2, the Sava River represents one of Europe's ecologically most interesting lifelines. As previously stated, several international surveys have been conducted on the Sava River Basin; yet, sponges have never been in the focus of investigations. In the Slovenian stretch of the Sava river there were no sponges, in Serbia they were scarce (abundance level 1 on all sites), while in Croatia, near Županja, three species were present (abundance level 3).

In addition to the Ramsar Convention document (2008) and the exhaustive Sava River monograph (Milačič, 2015), where sponges are only mentioned, this study represents the first screening of freshwater sponges in Serbia, and it appears that they are infrequent and their diversity limited. This is not an uncommon phenomenon in European running and still waters: in the Ebro River Basin (Spain), only *E. fluviatilis* and *E. fragilis* were recorded (Oscoz *et al*., 2009), and in the Temo River (Sardinia, Italy) *E. fluviatilis* was the single species collected (Manconi and Pronzato, 1994; Cubeddu *et al.*, 1995). Interestingly, in the Rhine during the seventies only *E. fluviatilis* could be found, although beyond that period, other species were also present (Gugel, 2001). Similarly, in the Serbian portion of the Danube, *E. fluviatilis* was the sole registered species. Remarcably, *T. horrida* was found at one site only in the Romanian portion of the Danube, which is in sharp contrast with the Volga river, where it represents the most common sponge (Schletterer, 2006). Based on physico-chemical data, sponges showed preference toward slightly alkaline and well oxygenated water, higher water temperatures and conductivity, higher concentrations of NO_3, and lower concentrations of PO_4. This is generally in line with some previous studies (Richelle-Maurer *et al.,* 1994). It has also been shown that sponges may grow in relatively polluted water which is the case with Sava and Danube. Levels of anthropogenic pollution vary considerably along the river courses, but it is considered that the permitted concentrations of heavy metals are usually not exceeded. Even in heavily industrialized zones with poor wastewater treatment, Intervention Values were not reached (Antonijević MD *et al.,* 2013). The presence of pollutants might account, among other, to spicule malformations found in sponges of both rivers at all inspected sites, without striking difference in malformation number between the sites.

CONCLUSIONS

Based on this study, one of the few dealing with sponges in the Danube and the Sava, both rivers are characterized by a rather low abundance of porifera with a limited diversity of species.

Further studies on a larger scale, possibly collected in a wider time-window, are needed for a more reliable overview of the distribution of these organisms in Danube and Sava rivers.

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Tab. 1. Comparison of minimum, maximum and average values of measured physical and chemical parameters between all localities and localities with sponges in the Sava and the Danube rivers.

Danube River	**General**			**Sponge localities**		
Physical and chemical parameters	Min	Max	Average	Min	Max	Average
pH	6.73	9.03	8.1	7.89	8.26	8.09
t (C°)	16.89	24.2	20.86	19.08	22.08	21.04
conductivity (µS)	195.4	1121.9	417.17	375.3	497.9	411.76
O_2 (mg/l)	4.26	10.54	8	5.89	9.02	7.87
NO_3(mg/l)	0.16	23.03	6.11	4.22	12.43	6.46
PO_4(mg/l)	0.1	1.54	0.21	0.13	0.23	0.16
Sava River	**General**			**Sponge localities**		
Physical and chemical parameters	Min	Max	Average	Min	Max	Average
pH	7	8.94	7.75	7	8.94	7.8
t (C°)	9.9	24.8	21.1	22.4	24.8	23.2
conductivity (µS)	194	587	336	194	587	359
O_2 (mg/l)	5.48	10.5	8.01	6.23	9.94	7.7
NO_3(mg/l)	1.44	6.69	3.71	2.04	4.56	3
PO_4(mg/l)	0.024	0.372	0.137	0.087	0.372	0.154

Tab. 2. Details of collected species and GenBank accession numbers sequences of the used freshwater sponges for 28S rDNA.

Taxon	Locality	Specimen code	GenBank Accession #
Family Spongillidae			
Spongilla lacustris	Serbia; Sava		
Spongilla lacustris	Serbia; Sava	AS37	
Spongilla lacustris	Croatia, Sava	AS40	
Spongilla lacustris	Croatia, Sava	AS41	
Spongilla lacustris	Serbia; Sava	AS46	
Spongilla lacustris	Serbia; Sava	AS48	
Spongilla lacustris	Estonia		DQ454154
Eunapius fragilis	Serbia; Sava	AS44	
Eunapius fragilis	Serbia; Sava	AS45	
Eunapius fragilis	Estonia		DQ454155
Ephydatia fluviatilis	Serbia; Danube	AS30	
Ephydatia fluviatilis	Serbia; Danube	AS31	
Ephydatia fluviatilis	Serbia; Danube	AS32	
Ephydatia fluviatilis	Serbia; Danube	AS33	
Ephydatia fluviatilis	Serbia; Danube	AS34	
Ephydatia fluviatilis	Serbia; Danube	AS35	
Ephydatia fluviatilis	Serbia; Sava	AS38	
Ephydatia fluviatilis	Croatia, Sava	AS39	
Ephydatia fluviatilis	Croatia, Sava	AS42	
Ephydatia fluviatilis	Croatia, Sava	AS43	
Ephydatia fluviatilis	Croatia, Sava	AS46	
Ephydatia fluviatilis	Estonia		DQ454152
Ephydatia fluviatilis	Italy		EF591285
Ephydatia fluviatilis	Israel		JN116226

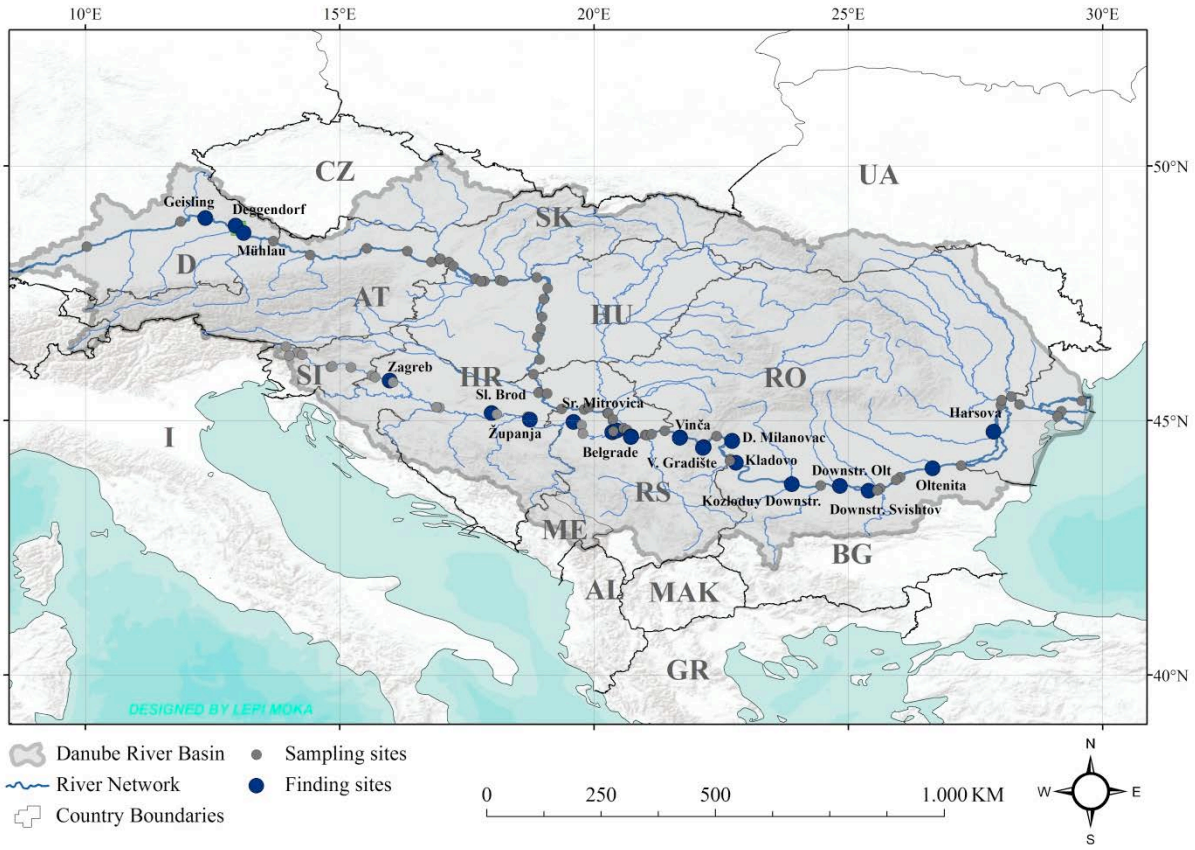

Fig. 1. Approximate distribution of freshwater sponges collected in the framework of Joint Danube Survey 3 investigation (JDS 3, International Commission for the Protection of the Danube River, 2013) and GLOBAQUA project (Navarro-Ortega *et al*., 2015) in the period 2013-2015.

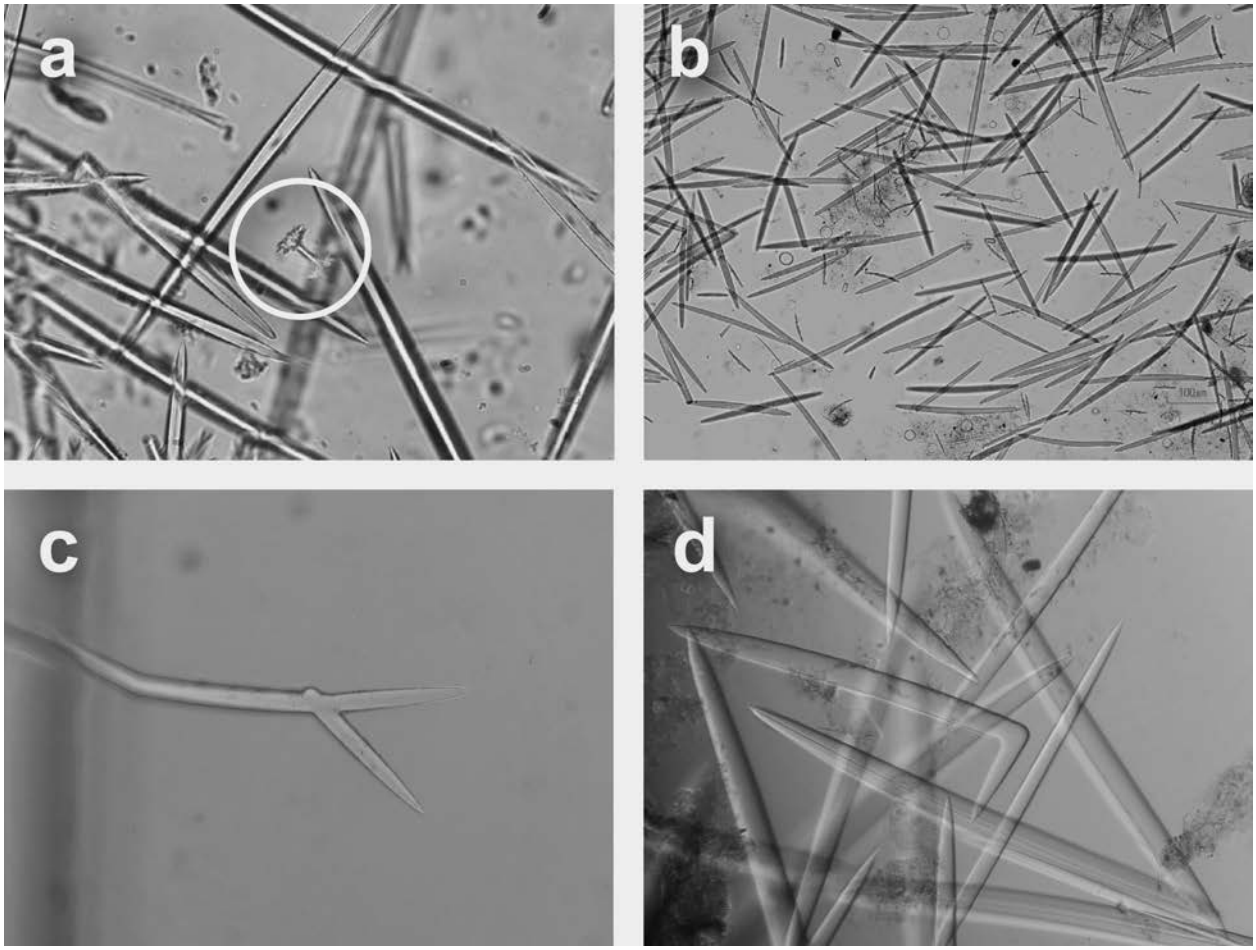

Fig. 2. a) Typical skeletal structures of *Ephydatia fluviatilis:* long and smooth monaxial oxea (megascleres) and birotule (gemmulosclere) with characteristic starshaped rotules and shaft longer than the width of the rotules. b) Typical skeletal structures of *Spongilla lacustris*: smooth oxea (megascleres) and thorny microscleres and gemmuloscleres. c,d) Representative spicules with anomalies.

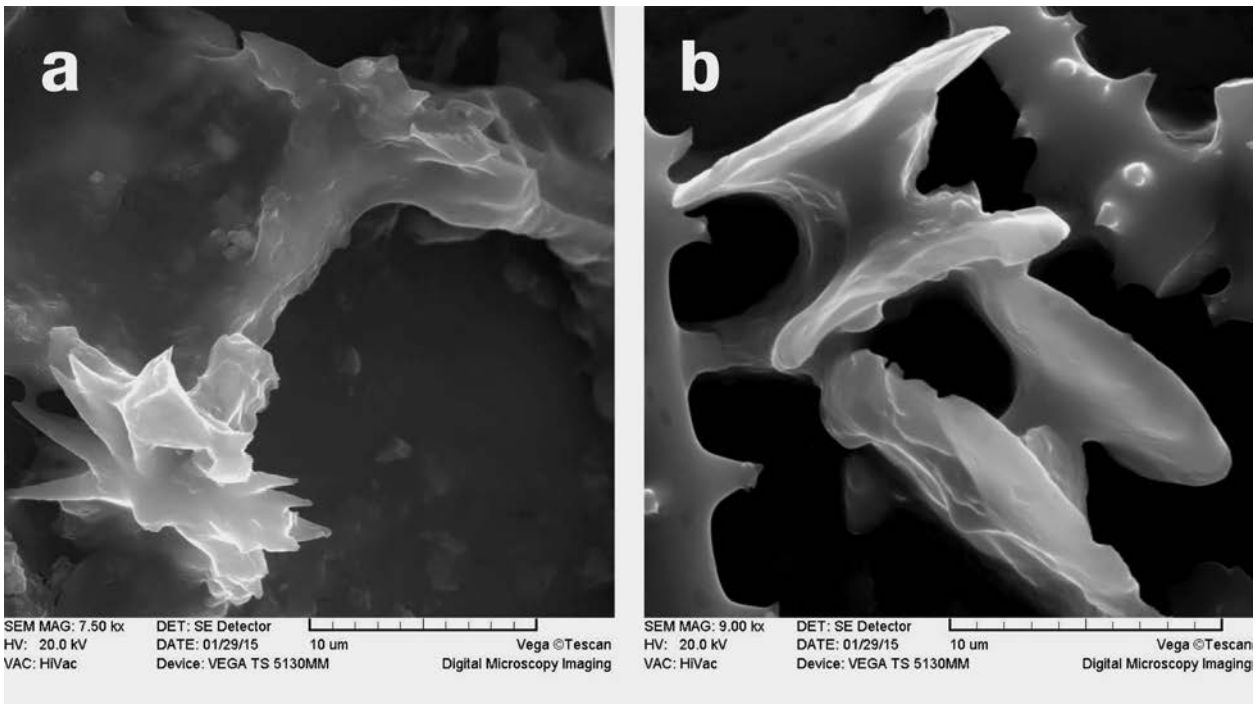

Fig. 3. SEM images of: a) *E. fluviatilis* gemmulosclere (birotule); b) two birotules of *T. horrida*.

Fig. 4. Phylogenetic trees based on the D3 domain of 28S rDNA obtained using the Neighbor-Joining (NJ) method. Bootstrap values are indicated below the branches. The scale bar indicates the number of substitutions per site.